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Review:

The development of targeted new agents to improve the outcome for children with leukemia

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Abstract

Introduction: Survival rates in pediatric leukemia have greatly improved in the last decades but still a substantial number of patients will relapse and die. New agents are necessary to overcome the limitations of conventional chemotherapy and hematopoietic stem cell transplantation and to reduce their undesirable long-term toxicities. The identification of driving molecular alterations of leukemogenesis in subsets of patients will allow the incorporation of new-targeted therapies.

Areas covered: In this article the authors present a detailed review of the most recent advances in targeted therapies for pediatric leukemias. A comprehensive description of the biological background, adult data and early clinical trials in pediatrics is provided.

Expert opinion: Clinical trials are the way to evaluate new agents in pediatric cancer. The development of new drugs in pediatric leukemia must be preceded by a solid biological rationale. Agents in development exploit all possible vulnerabilities of leukemic cells. Drugs targeting cell surface antigens, intracellular signaling pathways and cell cycle inhibitors or epigenetic regulators are most prominent. Major advances have occurred thanks to new developments in engineering leading to optimized molecules such as anti-CD19 bi-specific T-cell engagers (e.g. blinatumomab) and antibody-drug conjugates. The integration of new-targeted therapies in pediatric chemotherapy-based regimens will lead to improved outcomes.

Abbreviations

Term	Abbreviation
Accelerated phase	AP
Acute lymphoblastic leukemia	ALL
Acute myeloid leukemia	AML
Acute promyelocytic leukemia	APL
Adverse event	AE
Antibody-drug conjugate	ADC

All-trans retinoic acid	ATRA
Azacitidine	AzaC
Berlin-Frankfurt-Münster	BFM
bi-specific T-cell engager	BITE
Bis in die (Twice daily)	BID
Blastic phase	BP
Burkitt lymphoma	BL
Central nervous system	CNS
Cerebrospinal fluid	CSF
Children Oncology Group	COG
Chimeric antigen receptor T	CAR-T
Chronic lymphocytic leukemia	CLL
Chronic myeloid leukemia	CML
Chronic phase	CP
Complete remission	CR
Complete remission with partial hematologic recovery	CRh
Complete cytogenetic response	CCyR
Cytokine release syndrome	CRS
Down syndrome	DS
DNA methyltransferase	DNMT
International Berlin-Frankfurt-Münster Study Group	iBFM-SG
European Medicines Agency	EMA
Experimental Cancer Medicine Centre	ECMC
Event-free survival	EFS
Gemtuzumab-ozogamicin	GO
Hematopoietic stem cell transplantation	HSCT
Innovative Therapies for Children with Cancer	ITCC
Internal tandem duplication	ITD
Intravenous	IV
Juvenile myelomonocytic leukemia	JMML
Lymphoblastic lymphoma	LL
Major cytogenetic response	MCyR

Major histocompatibility complex	MHC
Major molecular response	MMR
Maximum tolerated dose	MTD
Minimal residual disease	MRD
Mitogen-activated protein kinase	MAPK
Mixed lineage leukemia	MLL
Mixed lineage leukemia rearranged	MLLr
Monoclonal antibody	mAb
Monomethyl auristatin F	MMAF
Myelodysplastic syndrome	MDS
Once daily	OD
Overall survival	OS
Pediatric Preclinical Testing Program	PPTP
Pharmacokinetics	PK
Polo-Like kinase 1	PlK1
Progression-free survival	PFS
Recommended phase II dose	RP2D
Relapse-free survival	RFS
Rhabdomyosarcoma	RMS
Stable disease	SD
Therapeutic Advances in Childhood Leukemia	TACL
Therapeutically Applicable Research to Generate Effective Treatments	TARGET
Tyrosine kinase domain	TKD
Tyrosine-kinase inhibitor	TKI
United Kingdom	UK
United States	US
Veno-occlusive disease	VOD
Wild-type	WT

Article highlights

- Leukemia is the most frequent type of cancer in children. Survival rates have improved in the last decades but still a substantial number of patients will succumb to their disease. New agents are necessary to overcome the therapeutic imitations of conventional chemotherapy and hematopoietic stem cell transplantation and to reduce their long-term toxicity.
- Pediatric research platforms for the identification of molecular alterations and novel targeted therapies have been developed, such as the Pediatric Pre-clinical Testing Program and the Therapeutically Applicable Research to Generate Effective Treatments project in the United States or the Innovative Therapies for Children with Cancer Consortium in Europe. A close collaboration between pharma, regulators and academic groups is necessary to incorporate these therapies into clinical practice.
- New agents have been developed to target pathogenic molecular alterations at different cell levels: cell surface receptors, tyrosine-kinases, signaling pathways and the cell cycle. Immunotherapy in leukemia is key and new promising agents are being developed such as antibodies or CAR-T cells.
- The introduction of targeted agents in particular subsets of children with leukemia harboring individual molecular alterations has radically changed their outcome, such as BCR-ABL fusion gene in chronic myeloid leukemia and Ph+ acute lymphoblastic leukemia. The identification of driving targetable molecular alterations in clonal leukemic cells will allow individualizing treatments while sparing patients of undesirable side effects.

1. Introduction

Leukemia is the most common type of cancer in children [1]. 90-95% of those are acute leukemias. Acute lymphoblastic leukemia (ALL) is the most frequent (75-80%), followed by acute myeloid leukemia (AML) (20%) [1]. Less frequent are chronic myeloid leukemia (CML) and juvenile myelomonocytic leukemia (JMML) [1].

The optimization in the use of existing conventional chemotherapeutic anti-leukemic agents together with improvements in risk-group stratification and supportive care have led to a significant increase in cure rates. Five-year event free survival (EFS) in pediatric ALL has increased from less than 10% in earlier efforts to more than 80% with current front-line regimens [2]. With very few exceptions, the drugs used today were available by the late 1960s. This improvement has been largely achieved as a result of international collaborative efforts through clinical trials [3]. In AML five-year survival rates reach up to 76% [4].

Nonetheless, it seems that further intensification of treatment beyond the current standards is now maximized, and improvements in outcome have plateaued over the past decade [5]. Still, a substantial number of these patients will not be cured, and for those who survive long-term toxicities are of major importance [6]. Current efforts focus in particular clusters of patients with high-risk molecular or cytogenetic features, infants and relapsed or refractory leukemias [3].

Molecular targeted agents have been developed in recent years in adult oncology, aiming at pathways that function predominantly in leukemic cells, ideally with absent or minimal function in healthy tissues. Such pathways may be initiated by cell surface receptors (CD33, CD22), specific intracellular kinases (FLT-3; BCR-ABL); proteins regulating cell death (BCL-2 family), and modulators of gene function [7]. However, in pediatric leukemia no other targeted agents have reached clinical practice besides tyrosine kinase inhibitors (TKI) in BCR-ABL rearranged ALL and CML.

The development of new agents in pediatrics virtually always lags behind that of the adults. Some reasons are: i) The success of frontline and even second line treatments results in a decreased number of children potentially able to participate in phase I-II trials; ii) The relatively small market of pediatric oncology does not provide the financial incentives for companies to actively pursue pediatric oncology drugs [8]; iii) Cancer biology in children is different from that of the adults in some diseases [9]; iv) Scarcity of preclinical pediatric models that allow to test broad-spectrum agents or histotype-specific activity against childhood cancers; v) Lack of incentives to groups developing new agents against specific pediatric targets [10]. Pure pediatric research platforms for the identification of molecular alterations and novel targeted therapies have been developed, such as the Pediatric Preclinical Testing Program (PPTP [11]; www.ncipptc.org)and the Therapeutically Applicable Research to Generate Effective Treatments project (TARGET [12]; target.nci.nih.gov) in the United States (US) and the Innovative Therapies for Children with Cancer (ITCC) consortium in Europe. These platforms are able to identify new targets and drugs that can be taken into clinic in collaboration with pharma and academic groups like the Therapeutic Advances in Childhood Leukemia (TACL), Children´s Oncology Group (COG), International Berlin-Frankfurt-Münster Study Group (iBFM-SG) or IntReALL.

In this manuscript we will review the progress made in the development of targeted agents in pediatric leukemia. Drugs selected for discussion have been chosen based on their pre-clinical data, mode of action, relevancy in pediatric leukemias and current degree of development (Figures 1 and 2). Table 1 shows active and near future clinical trials with novel agents. A critical summary highlighting those agents with most prominent results is provided in the Expert Opinion section.

2. Cell surface antigens: Immune strategies

This section summarizes most recent developments of agents targeting cell surface antigens of leukemic blasts, which exert a direct cytotoxic effect or trigger an indirect immune response.

2.1. Chimeric antigen receptors

First generation chimeric antigen receptors (CARs) are hybrid receptors that comprise a ligand for a cell-surface molecule, usually a single-chain variable fragment from a monoclonal antibody (mAb) or an antigen-binding fragment, which is fused to signaling domains redirecting T-cell function [13]. Second and third generation CARs incorporate additional domains to supply co-stimulatory signals enhancing T-cell toxicity [13].

CD19 CAR-T cells have yielded impressive results in adult chronic lymphocytic leukemia (CLL) with an overall response rate in the phase I trial approaching 60% in a heavily pretreated population [14].

In children, two landmark early trials have shown exciting results in B-ALL creating major enthusiasm and social and media interest [15]. In pediatric trials, complete response (CR) rates ranged from 70 % to 90% [16, 17] and minimal residual disease (MRD) negativity rates of around 85% among those who achieved CR. In the Lee study, of those MRD negative patients, ten were able to proceed to hematopoietic stem cell transplantation (HSCT) and were still alive at last follow-up [17]. Interestingly, patients with central nervous system (CNS) disease cleared cerebrospinal fluid (CSF) blasts after treatment [16, 17] and no CNS relapses were observed [16]. Importantly, the feasibility rate of producing CD19 CAR-T cells was high (90%) [17]. The toxicity profile was mainly represented by the cytokine release syndrome (CRS) and hematological toxicities related to the lympho-depleting regimen.

A phase II trial of CD22 CAR-T cells is open for pediatric patients with relapsed/refractory CD22-expressing B cell malignancies (NCT02315612).

2.2 CD20 directed therapy

The surface antigen CD20 is present in 100% of children and adolescents with Burkitt lymphoma (BL) and 40% with B-ALL [18]. CD20 influences cell cycle progression and differentiation by modulating levels of proapotosis proteins and activation of surviving pathways [19].

The most consolidated agent in this group is the chimeric mAb rituximab. Despite being widely used in adults and children, it still does not have a specific label for pediatric use. It has been used in combination with the hyper-CVAD regimen, as CD20 expression had been associated with higher relapse rates in adults with the novo B-cell ALL [20]. This combination significantly improved survival in adolescents and young adults with newly diagnosed CD20 B-ALL when compared with chemotherapy alone (3-year overall survival (OS) 75% Vs 47%) [19]. In pediatric high-risk B-mature acute leukemia and BL, addition of rituximab to conventional chemotherapy significantly improved their outcome: 1-year EFS was 94.2% Vs 81.5% for those not receiving rituximab [21].

Ofatumumab targets a different epitope than rituximab. In adults with CD20 ALL, ofatumumab was added to the hyper-CVAD regimen. The 1-year PFS and OS were 91% [22]. Most common grade 3 toxic events were hepatic, thrombosis and neuropathy. One trial is currently evaluating ofatumumab in newly diagnosed or first relapse B-cell ALL or lymphoblastic lymphoma (LL) children and adolescents with augmented BFM chemotherapy (NCT02419469).

2.3 CD19 directed therapy

CD19 is expressed ubiquitously on B cells from the pro/pre B-cell stage to mature B-cells and their malignant counterparts [23]. CD19 sustains the malignant B-cell phenotype via mechanisms of proliferation, cell survival and self-renewal [24].

Blinatumomab is a bi-specific CD19-directed CD3 T-cell engager (BITE) facilitating the activation of endogenous T cells when bound to the CD19 expressing target cell regardless of the T cell receptor specificity or reliance on major histocompatibility complex (MHC) class I molecules on the surface of antigen presenting cells for activation [24]. This circumvents a known mechanism of resistance to T cell therapies through down-regulation of MHC class molecules. Blinatumomab was initially given as short IV infusions, but the absence of objective responses and the toxicity profile represented by CNS toxicities, CRS and infections, prompted its investigation as continuous infusion [24]. Initial phase II studies showing significant response rates in heavily pretreated patients led to considerable excitement and a conditional approval by the European Medicines Agency (EMA) in 2015 for adult relapsed ALL. A single arm phase II adult study in relapsed ALL demonstrated a CR rate of 33% (n=63) and CR with partial hematologic recovery (CRh) of 10% (n=18) after two cycles [25]. After a median follow up of 8.9 months, 37% of those eighty-one patients achieving CR/CRh were alive and in remission.

Blinatumomab in children has been investigated in thirty-nine patients with refractory or relapsed B-ALL as continuous IV infusion for 4 weeks up to 5 cycles [26]. During the first two cycles, twelve patients (31%) achieved CR, and of those five had complete MRD response. Six out of these twelve patients underwent bone marrow transplantation. Median relapse-free survival (RFS) for responding patients was 5.6 months. Most common severe adverse events (AEs) were anemia, pyrexia, increased

ALT/AST and febrile neutropenia. Grade 3 CRS was reported in two patients (5%). In Europe, a phase III trial (NCT02393859) will randomize high-risk relapsed B-cell ALL patients to receive blinatumomab or a conventional chemotherapy consolidation block before transplantation in a first step. In the adaptive phase of the study, patients will be randomized to receive blinatumomab or three consolidation chemotherapy blocks before transplant. In the US a phase III trial (NCT02101853) randomizes high and intermediate-risk relapsed B-cell ALL between blinatumomab and conventional chemotherapy following induction and low-risk patients between chemotherapy +/- blinatumumab following induction.

Coltuximab Ravtansine (SAR3419) is an anti-CD19 humanized mAB drug conjugate to DM4, a potent antimitotic agent. Coltuximab is active in pediatric models of B-ALL and infant mixed lineage leukemia (MLL) including chemo resistant Ph+ ALL [27]. In the phase I-II study of coltuximab single agent in relapsed B-ALL thirty-six adult patients were treated with manageable toxicities [28]. The recommended phase II dose (RP2D) was 70mg/m^2 given IV once weekly for 8 weeks.

Denintuzumab Mafodotin (SGN-CD19A) is a novel antibody-drug conjugate (ADC) composed of a humanized anti-CD19 mAb conjugated to the microtubule-disrupting agent monomethyl auristatin F (MMAF), which binds to tubulin and induces G2/M arrest and apoptosis [29]. In the phase I study of denintuzumab single agent in relapsed B-ALL seventy-one adult patients participated [30]. Maximum tolerated dose (MTD) was 5mg/kg every 3 weeks and the MTD was not reached with the weekly dosing. The combined CR rate was 19% for the weekly dosing and 35% for the 3-weekly. AEs were similar with both schemas: gastrointestinal, ocular and hematological. The promising results in the Ph+ group have prompted an expansion cohort on the 3-weekly dosing.

2.4 CD22 directed therapy

CD 22 is expressed in more than 95% of B-cell ALL in children [31]. CD22 shifts from the cytoplasmic domain in developing B cells to the cell surface in later stages of B-Cell development.

Epratuzumab, a humanized anti-CD22 mAb, is internalized after binding to cell surface CD22 and modulates B-cell activation and signaling [32]. In the phase II COG study in ALL relapsed patients, epratuzumab was given once (B1) or twice weekly (B2) for a total of 4 or 8 doses during block 1 of re-induction [33]. CR rates after block 1 were similar for both cohorts and with the AALL01P2 study, a trial using the same chemotherapy (B1: 65%; B2: 66%; AALL01P2: 74%). Rates of MRD negativity were higher in this phase II study when compared with AALL01P2 (39% Vs 25%), results that should be interpreted with caution given the methodological difficulties of using historical controls. Early relapsed patients treated in cohort B2 had significant superior 2-year EFS when compared to AALL01P2 study (54.6% Vs. 25.7%,). IntReALL 2010 investigates the value of adding epratuzumab from induction to consolidation in a randomized fashion to two different regimens, the modified protocol ALL-REZ BFM 2002 and the UK ALL-R3 in standard risk relapsed ALL patients (NCT01802814).

Inotuzumab ozogamicin is a humanized mAb directed at CD22 that conjugates the moAb with calicheamicin, an antitumor antibiotic. A phase I-II study evaluated two different schedules in relapsed/refractory ALL including children [34]. The overall response rate was 58% and the CR rate was 19%. A total of 40% of patients could undergo transplantation afterwards. In adults with relapsed ALL, inotuzumab single agent was challenged against three standard relapse regimens. The CR rate was significantly higher with inotuzumab (80.7% Vs 29.4%) and more patients receiving inotuzumab had results below the MRD threshold (78.4% Vs 28.1%) [35]. Most

frequent toxicity was veno-occlusive disease (VOD). An ITCC phase I-II study evaluating inotuzumab in children with refractory/relapsed ALL is launching this year in Europe (EudraCT 2016-000227-71).

Moxetumumab pasudotox is a recombinant immunoconjugate composed of an anti-CD22 immunoglobulin variable domain genetically fused to a truncated form of *Pseudomonas* exotoxin [36]. A phase I trial single agent in children and young adults with relapsed CD22+ hematological malignancies was associated with an acceptable toxicity profile (mainly hepatic). Objective responses were achieved in eleven patients (30%) including 9 (24%) CR [36]. A recent phase II study in pediatric relapsed ALL and LL has been early terminated because the required efficacy for the study continuation was not met (NCT02227108). Possible explanations for this limited activity are that higher doses are likely required to achieve maximal benefit and that drug exposure may be limited to rapid clearance [37]. To overcome these limitations, less immunogenic formulations that can be given at higher doses [38] and the combination with protein kinases enhancing its activity [39] are investigated.

2.5 CD33 directed therapy

The CD33 cell surface antigen is present in more than 80% of patients with AML [40]. Gemtuzumab-ozogamicin (GO) is a humanized anti-CD33 antibody linked to the DNA-binding cytotoxin calicheamicin. Single agent activity was seen in a pediatric phase I study for relapsed AML with CR rates of 28% [41]. *De novo* AML children enrolled in the phase III COG trial AAML0531 were randomly assigned to receive or not GO during induction and consolidation [42]. Three-year EFS was significantly better in the GO group (53.1% Vs 46.9%) but there was not significant effect on OS (69.4% Vs 65.4%). FLT3/Internal Tandem Duplication (ITD)-positive *de novo* AML pediatric

patients treated with GO and conventional chemotherapy and HSCT in first remission experienced less relapses in two consecutive COG trials [43]. GO may also have a role in the post-transplant setting as a consolidation regimen for those patients with AML, where its use has proved to be safe [44] and it is now investigated in a larger population (NCT02117297) where GO is given from day 60 to 180 post-transplant. In adults the addition of GO to daunorubicin and cytarabine resulted in a significantly higher fatal induction toxicity rate (mainly hemorrhages, VOD and other G4 non-hematological toxicities) [45]. This prompted its withdrawal from the US market in 2010 [46]. Nonetheless, the interest for GO has been revived in AML after two large trials in newly diagnosed adult AML showed improvements in OS [47, 48]. An international phase III trial will randomize newly diagnosed AML pediatric patients to receive or not GO with conventional chemotherapy (NCT02724163). The key point is to find a balance between toxicity and efficacy. Low doses of $3mg/m^2$ can have anti-leukemic effect and less undesirable side effects [49].

In acute promyelocytic leukemia (APL) CD33 is expressed in virtually 100% of APL cells and GO has been better tolerated than conventional chemotherapy, particularly in older patients [50]. GO is currently under investigation in newly diagnosed APL in combination with ATRA and arsenic trioxide (NCT01409161).

SGN-CD33A is a novel ADC with a similar antileukemic activity to that of GO but without liver toxicity [51]. AMG 330 is a T-specific-cell-engaging antibody with dual specificity for CD3/CD33 that has shown promising activity in preclinical models and has now entered first in human clinical trials [52].

3 BCR-ABL inhibitors

The introduction of the TKI imatinib in front-line Ph+ ALL pediatric protocols radically changed the prognosis of these patients. Continuous use of imatinib along with conventional chemotherapy has improved the EFS of these patients from 29% to 81% [53]. In the COG-ALLL0031 study, the positive impact of continuous imatinib persisted regardless of the whether these patients were treated only with chemotherapy or received an allogeneic transplant [54]. A major drawback is the appearance of resistance, often through the development of point mutations in the ABL tyrosine kinase domain (ABL-TKD) [55]. Second generation TKI such as dasatinib and nilotinib are effective for M244V and H396P mutations [56] but not for T315I; dasatinib has been investigated in children with newly diagnosed Ph+ ALL (NCT00720109). Ponatinib is highly effective in T315I mutated Ph+ ALL [57], although severe vascular side effects can occur [58]. Pediatric development for ponatinib has not started yet.

In pediatric CML, TKIs have become the cornerstone of first-line treatment. Children with newly diagnosed CML in chronic phase (CP) treated with imatinib 260mg/m^2 [59] had a 3-year PFS of 98%. The rates of complete cytogenetic response (CCyR) and major molecular response (MMR) were 77% and 57% during follow up.

In the ITCC phase I study of dasatinib, of the 17 patients with CML-CP, 94% had a CHR, 88% major cytogenetic response (MCyR) and 82% CCyR [60]. PFS and OS at two years were 61% and 88%. The RP2D in children with CP-CML was 60mg/m^2 OD and 80mg/m^2 for those with accelerated phase (AP)/blastic phase (BP)-CML or Ph+ ALL. The pediatric study with nilotinib for children with newly diagnosed and resistant or intolerant to imatinib/dasatinib CML has recently completed recruitment (NCT01844765). An ITCC phase I/II study evaluating bosutinib in pediatric patients with refractory/relapsed or intolerant to TKIs CML is launching this year in Europe

(Netherlands trial registry number: NTR5501). Most frequent toxicities are hematological. Non-hematological toxicities are rash, edema, hepatic, myalgia, bone pain, growth retardation and QT prolongation [59–61].

4 Small molecule inhibitors of intracellular kinases

This section will cover agents targeting intracellular signaling pathways mediated by kinases activated in leukemic cells or that form part of the cell cycle.

4.2 JAK/STAT

The JAK family of tyrosine kinases activates the STAT family of transcription factors. The JAK/STAT pathway mediates cytokine receptor-derived signals and plays a role in hematopoietic cell growth, proliferation, differentiation and survival [62].

In a series of fifty-three Down´s syndrome associated ALL (DS-ALL) patients, all ten patients with *JAKR683* somatic mutations had *CRLF2* aberrant expression [63]. Therapies blocking the CRFL2/JAK2 pathways are an attractive approach in these patients.

In a series of pediatric T-cell ALL, 45% had mutations in *IL7Ra*, *JAK, RAS*, *AKT* and *PTEN* and occurred in a mutually exclusive fashion suggesting that they share aberrant activation of similar downstream targets [64]. *IL7Ra* and *JAK* mutants were relatively resistant to downstream RAS-MEK-ERK or PI3K-AKT-mTOR inhibition, suggesting that a combined synergistically inhibition can be of interest.

In children with Ph-like ALL, genetic alterations and rearrangements in *CRLF2*, *JAK-STAT*, *ABL1* and *PDFGRB* have been frequently observed, with up to 50% in the case of *CRLF2* rearrangements [65].

In the phase I trial of the JAK inhibitor ruxolitinib in children with relapsed cancers the RP2D was 50mg/m^2 orally BID continuously [66] but no responses were seen in patients with leukemia. Only one patient with polycythemia vera achieved partial response. There were not *JAK* aberrant cases. The inhibition of phosphoproteins JAK2, STAT5 and S6 was not dose dependent. Most frequent toxicities were hematological and gastrointestinal.

A COG phase II study is now evaluating ruxolitinib in combination with chemotherapy in newly diagnosed high-risk Ph-like B-ALL patients (NCT02723994).

4.3 Ras/Raf/MEK/ERK

The mitogen-activated protein kinase (MAPK) cascade regulates cell proliferation and survival [67] and generates signal output through other effector pathways such as PI3K/AKT/mTOR and RalGEF/RAL [68]. Involved mechanisms in the activation of the Ras pathway include somatic mutations in upstream activators or regulatory proteins (*NRAS*, *KRAS*, *BRAF*, *FLT3*, *PTPN11*, *CBL* or *NF1*) and the incidence of these mutations in newly diagnosed ALL is up to 35% and 39% in relapsed patients [68]. In a series of 206 relapsed ALL patients, *RAS* mutant positive patients had a higher proportion of early relapses and shorter median time to relapse compared with wild type (WT) patients [69]. *RAS* mutations are an independent predictor for poor outcome in *MLL* rearranged infant ALL[70]

Due to the difficulties in developing direct inhibitors of the RAS protein itself, efforts have focused on disrupting RAS post-translational processing. In this sense, tipifarnib, an orally bioavailable farnesyltransferase inhibitor, was tested in a phase I study including children with refractory leukemias [71]. The RP2D was established at 300mg/m^2 BID for 21 days of a 28-day cycle. No responses were seen in ALL patients

and only one patient with JMML experienced stable disease (SD). Toxicities were mainly cutaneous and gastrointestinal.

MEK inhibition is an attractive approach in pediatric ALL, as it may be capable to inhibit the pathway regardless of the mechanism of upstream activation. The frequency of mutations in the RAS/RAF/MEK pathway is higher in prednisolone-resistant ALL-children [72]. A complete sensitization to prednisolone after trametinib was observed in pediatric ALL cell lines, suggesting that MEK inhibition may modulate prednisolone resistance and improve clinical outcome of childhood B-ALL [72]. Selumetinib induced dramatic reduction in leukemia cells in mice with implanted *RAS* mutant ALL [69]. Single agent activity was modest in untreated adult AML, but with favorable toxicity profile, suggesting its potential role in combination [73].

4.4 FLT3 inhibitors

Activating mutations of the *FLT3* gene have been described in pediatric ALL and AML, particularly in those with 11q23/MLL rearrangements [74]. Lestaurtinib, an FLT3 inhibitor, was found to be highly active in ALL cell lines with MLL gene rearrangements, high-hyperdiploidy and FLT3 mutations [75]. An ongoing phase III study for infants with newly diagnosed *MLL* rearranged ALL evaluates the addition of lestaurtinib in a randomized fashion to combination chemotherapy (NCT00557193). Midostaurin single agent has been evaluated in a phase I/II trial in pediatric patients with relapsed/refractory leukemia [76]. The RP2D for combination studies is 30mg/m^2 BID. Five patients with AML and three ALL achieved a modest clinical response. Most frequent toxicities were vomiting, pyrexia and thrombocytopenia. Crenolanib is currently being investigated in combination with sorafenib in patients with relapsed/refractory AML and FLT3-ITD/TKD mutations (NCT02270788).

Sorafenib single agent and in combination has demonstrated to be active in pediatric AML mutant *FLT3/ITD* [77]. In the ongoing phase III COG AML trial (NCT01371981), high-risk *FLT3/ITD*+ patients receive sorafenib from induction.

4.5 Polo like kinases

Polo-Like Kinase 1 (PlK1) is a serine/threonine specific kinase implicated in several steps of cell mitosis [78]. Volasertib inhibits PlK1, resulting in cell cycle arrest and apoptosis [78]. Pre-clinical activity has been demonstrated in ALL, rhabdomyosarcoma (RMS), neuroblastoma and glioblastoma, both as single agent [78] and in RMS with chemotherapy [79]. A phase I dose escalation trial in refractory/relapsed pediatric tumors has been recently completed (NCT01971476). A phase I trial in children with AML after front line failure combines volasertib with chemotherapy (NCT02722135).

5. Proteasome inhibitors

Proteasome inhibitors have emerged as a one of the most promising therapeutics for hematological malignancies, particularly in adult multiple myeloma [80]. Pre-clinical studies indicate that malignant cells are more susceptible to their cytotoxic effects than normal cells. Several cellular processes contribute to their apoptotic effect: inhibition of NFκB activity, altered degradation of cell cycle related proteins, altered pro-apoptotic and anti-apoptotic protein balance, endoplasmic reticulum stress and inhibition of angiogenesis and DNA repair [80].

Bortezomib, the first proteasome inhibitor authorized for multiple myeloma in adults, demonstrated activity in ALL cell lines and xenografts models [81]. The phase I trial of bortezomib with standard induction chemotherapy in children with relapsed ALL [82] defined the RP2D was 1.3mg/m^2 on days 1, 4, 8 and 11 and 6/10 patients achieved a

CR. This schema was evaluated in a phase II study with 22 patients [83] and an 80% response rate in B-precursor patients. The COG evaluated the safety of bortezomib in combination with either idarubicine/cytarabine or cytarabine/etoposide in a cohort of forty-six patients with relapsed AML [84]. The CR rates were 29% and 43% respectively. Toxicity is mainly hematological, infection and neuropathy. Toxicity on thrombopoiesis from bortezomib has been observed in adults with advance hematological malignancies and multiple myeloma [85] that may limit its development in certain hematological diseases, which does not seem to be such a frequent event in children [83].

Bortezomib is under investigation in the phase III COG trial for newly diagnosed T-Cell ALL and lymphoma (NCT02112916) where patients are randomized to receive it or not during induction. In the European phase III BFM study in high-risk first relapsed ALL (EudraCT: 2012-000810-12), patients will be randomly assigned to receive or not bortezomib during induction. In AML, the phase III COG AML trial (NCT01371981) is currently randomizing newly diagnosed low and high-risk patients to receive or not bortezomib with combination chemotherapy.

Carfilzomib, a second-generation proteasome inhibitor, is under investigation in a pediatric phase I trial in relapsed ALL with conventional chemotherapy during induction (NCT02303821).

6. Epigenetic targeting

This section will cover drug candidates against epigenetic targets that have been described to play a role in pediatric leukemias.

6.1 DOT1L

Rearrangements in the *MLL* gene are present in up to 70% of patients with infant ALL [86]. The t(10;11)(p12;q23) and t(10;11)(p12;q14) translocations, which encode respectively the MLL-AF10 and CALM fusion proteins, are recurrent chromosomal rearrangements observed in acute leukemias [87, 88]. MLL-AF10 and CALM fusion proteins interact with histone H3 lysine 79(H3K79)-specific methyltransferase DOT1l. Although DOT1L has not been found to be genetically altered in leukemia [89], the interaction of these and others MLL-fusion proteins with DOT1L can lead to the mistargeting of DOT1L and subsequent methylation of H3K79, favoring leukemogenic transformation [90, 91]. EPZ5676 (pinometostat), a small molecule inhibitor of DOT1L H3K79 methyltransferase activity, has shown activity in cell lines bearing MLL-AF9, MLL-AF4, and MLL-ENL fusions [92]. A phase I trial investigating EPZ5676 single agent in pediatric patients with relapsed leukemia harboring rearrangements of the *MLL* gene has included eleven patients [93], showing similar PK profiles between adults and children. No responses were observed.

Besides this, the BCL-2 anti-apoptotic proteins are utilized by the lymphoid malignancies to maintain viability under conditions of oncogenic stress [94]. Chromatin-sequencing studies have shown that MLL/AF4 up-regulates the *BCL-2* gene but not other *BCL-2* family members via DOT1L-mediated H3K79me2/3 [95]. The combination of ABT-199, a BCL-2 inhibitor, with DOT1L inhibitors (SGC0946 and EPZ5676) showed deep growth inhibition in t(4;11) cells ; a synergistic effect was also demonstrated when ABT-199 was combined with chemotherapy [95]. Targeting BCL-2 is an attractive approach for multiple hematological malignancies [96] and therefore ABT-199 is now being investigated in adult CLL, AML and other non-Hodgkin lymphomas.

6.2 Hypomethylating agents (DNMT)

DNA methyltransferase (DNMT)-inhibiting cytosine nucleoside analogues reduce methylation from promoter regulatory regions of tumor suppressor genes silenced by DNA methylation, which reactivates cell growth arrest and differentiation [97].

Azacitidine (AzaC) and decitabine have been effectively used in the treatment of adult AML [98].

In children, AzaC has been used in newly diagnosed and relapsed myelodisplastic syndrome (MDS) and JMML with promising and durable responses allowing patients entering HSCT in some cases [99, 100]. Two phase II clinical trials are evaluating AzaC in children with newly diagnosed and relapsed MDS and JMML (NCT02447666 and EudraCT 2010-022235-10).

In pediatric AML, AzaC in combination with amsacrine and etoposide was active in refractory patients with AML, with a CR rate of 53% [101]. Decitabine has been evaluated as single agent as well as in combination with cytarabine but is not being taken forward into larger pediatric studies.

6.3 HDAC

Histone deacetylase (HDAC) inhibitors enhance histone acetylation leading to transcriptional repression and epigenetic silencing [102]. HDAC activity is increased in pediatric ALL and AML [103]. The use of HDAC inhibitors in *MLL*-rearranged ALL has been shown effective [104]. Vorinostat was evaluated in a phase I study in children with refractory tumors [105]. No responses were observed in the cohort of hematological malignancies. Vorinostat is currently under investigation in combination with chemotherapy and bortezomib for *MLL*-rearranged pediatric leukemias (NCT02419755). In ALL cell lines, vorinostat reprogramed the aberrant gene

expression profile of relapsed blasts and the incorporation of decitabine led to reexpression of genes methylated and silenced at relapse [106]. These two drugs followed in combination with conventional chemotherapy have been applied in a pilot study including eight children [107]. Overall response rate was 46.2% and five patients could be transplanted. A TACL phase I trial of panobinostat single agent in pediatric patients with relapsed ALL and AML has been recently completed (NCT01321346).

7. Future directions

Over the last two years, major advances in the field of immunotherapy, mainly with the development of immune checkpoint inhibitors have occurred in several adult solid tumors including melanoma and lung cancer. Anti-PD-1 and anti-PD-L1agents are showing promising results in adult leukemia and lymphoma trials and there is great interest in the pediatric community to evaluate this class of agents in childhood leukemia and lymphoma.

CAR-T cell therapy has demonstrated to be very effective in early studies in relapsed ALL, particularly in a fragile population where most patients had already undergone multiple treatment lines including allogeneic transplantation. After its approval for adults, blinatumomab has now entered phase III trials in first relapse pediatric high-risk B-ALL to test whether it may substitute conventional chemotherapy and serve as a less toxic bridge for transplantation. Inotuzumab single agent has been shown to be significantly more effective than other standard regimens for relapsed ALL in adults, and a pure pediatric trial is launching this year in Europe.

New BCR-ABL inhibitors are due to overcome the limitations on inherent or acquired resistance and toxicity when used in a prolonged fashion. Ponatinib is the first TKI demonstrating to be effective in T315I mutant Ph+ ALL, although its potential serious

vascular associated events in children are a concern, lower doses may counteract for a better safety profile and bosutinib is expected to have a better safety profile.

FLT3 inhibition is a promising approach in *MLL* and FLT3-driven leukemias, a necessary driver of leukemogenesis particularly in infants. FLT3 inhibitors have a manageable toxicity profile and its capacity to be combined with already effective chemotherapeutics makes them very attractive. The addition of lestaurtinib to conventional chemotherapy in infants with ALL has already been investigated, and results still waited. Sorafenib is under investigation in newly diagnosed AML mutant *FLT3/ITD.*

Proteasome inhibitors are also well advanced in their development. Bortezomib has reached phase III trials in newly diagnosed ALL. The addition of these agents to already intensive and toxic induction to remission regimens remains a concern and future strategies need to take into consideration this premise.

8. Conclusions

The impressive improvement in survival rates in pediatric leukemia, particularly ALL, between the early 60´s and 90´s is the most representative successful story in pediatric cancer. There has been no parallel before that or since then, notably because it has been achieved by using almost the same conventional chemotherapeutics that were available half a century ago. Our goal now is to continue improving these survival figures while minimizing the undesirable side effects of conventional treatment. New targeted therapies will truly contribute to this objective.

Integrating these novel agents into existing chemotherapy regimens and managing the additive side effects in an otherwise fragile population is complex. We have definitely entered into a new and fascinating era. Unveiling cancer genetics and discovering the mechanisms of escape of tumor cells opens the door to a more adapted therapy that will

lead to improved outcomes for these patients.

9. Expert opinion

The development of new agents in pediatric leukemia and its incorporation into clinical practice remains challenging.

Key challenges and barriers are well known and, while not discussed in detail in this review, they are being addressed by academic researchers and collaborative studies. Mainly, these comprise: i) Pediatric research platforms are necessary to identify molecular targets and to evaluate new agents by means of a comprehensive and meaningful pre-clinical testing that selects promising agents to be developed into clinical practice; ii) A close collaboration between industry and academic groups is essential to implement these findings into clinical practice; iii) Molecular screening at the time of relapse by means of high-throughput technologies may be able to identify molecular targets in particular patients that potentially can benefit from more directed approaches, rather than exposing patients to drugs that will not derive any benefit if the molecular abnormality is not present; iv) Combinations of biological agents are being tested to identify possible synergies and to overcome potential escape mechanisms; v) Improvements in incentives for those drugs and targets specific of pediatric cancers and vi) Switching pediatric drug development from a model centered on adult conditions to another more based on the mechanism of action [108]. ALL and AML comprise the majority of pediatric leukemias and will most likely need different developmental pathways. While the pediatric AML field might benefit from biological similarities between adult and pediatric AML, pediatric and adult ALL represent different entities and will need different pre-clinical and clinical studies.

Despite these challenges not overcome yet, we have witnessed major progress over the past five years thanks to the completion of sequencing studies at diagnosis and relapse, identification of new targets such as epigenetics, and numerous agents being tested preclinically. Thus, promising targets and drugs have been identified and these have been taken forward into pediatric early clinical trials in leukemia. Conducting these trials is demanding in a frail population with multiply relapsed leukemia and have required major efforts from cooperative groups in both sides of the Atlantic. Despite no drugs reaching regulatory approval for pediatric indications with the exception of imatinib, still the number of drugs entering the clinic and those transitioning from early to late phase clinical trials has significantly increased in recent years. Moreover, a better understanding of cancer biology, recurrent genomic aberrations and mechanisms of disease has led to more robust preclinical "proof-of-concept" data packages supporting clinical development of agents for pediatric leukemia.

As shown in this manuscript, the number of agents that have been tested in early trials in pediatrics is quite significant, with very few of them showing convincing positive results leading to fast-tracked development into larger studies and frontline use. In this sense, agents in early trials have been developed against most hallmarks of cancer, hence exploiting all possible vulnerabilities of leukemic cells, their microenvironment and immune response in order to maximize the efficacy of new drugs and combinations. Among those, agents targeting cell surface antigens, intracellular signaling pathways and cell cycle inhibitors or epigenetic regulators are most prominent. Additionally, major advances have occurred thanks to new developments in engineering leading to optimized molecules such as BITEs, antibody-drug conjugates or CAR-T cells with improved pharmacological and immunological properties. In this sense, agents holding most promise comprise some targeted agents that have had outstanding results in early

trials such as CAR-T cells or BITEs that remain to be confirmed in larger studies and other drugs that have already reached frontline or randomized clinical trials such as bortezomib or gentuzumab ozogamycin.

In summary, having identified the challenges and established the basis for more efficient preclinical and clinical drug development for pediatric leukemias, numerous agents are moving ahead in its development for pediatric leukemia and it is hoped that new drugs will reach clinical practice in coming years.

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Table footnotes

Table 1. Active and near future clinical trials with novel agents in pediatric leukemia

Footnotes:

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; APL: Acute promyelocytic leukemia; ATO: Arsenic trioxide; ATRA: All-trans retinoic acid; BFM: Berlin-Frankfurt-Munich BLe: Burkitt leukemia; BLy: Burkitt lymphoma; CARs: Chimeric antigen receptors; CML: Chronic myeloid leukemia; CT: Chemotherapy; GO: Gemtuzumab-ozogamicin; DNMT: DNA methyltransferase; HDAC: Histone deacetylase; HR: High-risk; IP: Investigational product; IR: Intermediate-risk; ITD: Internal tandem duplication; JMML: Juvenile myelomonocytic leukemia; LL: Lymphoblastic lymphoma; LR: Low-risk; MDS: Myelodysplastic syndrome; MLLr: Mixed lineage leukemia rearranged; SR: Standard-risk; TKD: Tyrosine-kinase domain; TKIs: Tyrosine-kinase inhibitors; UK: United Kingdom; VSLI: vincristine sulfate liposome injection.

* These two studies are part of the same trial (NCT01371981)

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*** Perspective on how to improve drug development in pediatric cancer**

Figures footnotes

Figure 1. Mode of action of selected drugs for discussion

In bold, agents that have reached phase III trials or are considered highest priority for development.

HDAC: Histone deacetylase

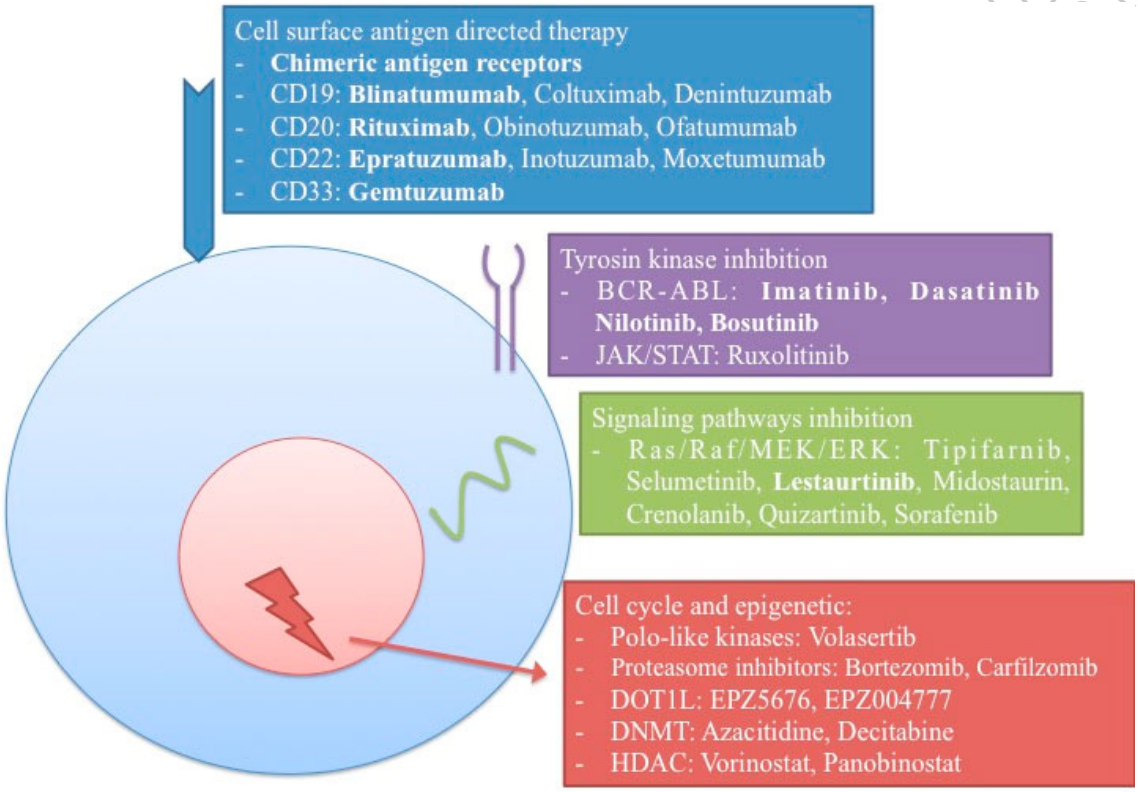

Figure 2. Current degree of development of selected drugs for discussion.

Most representative and advanced agents from each therapeutic group discussed in the manuscript are presented according to their respective degree of development. Top priority agents are highlighted in bold.

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; BITE: b-specific T-cell engager; CML: Chronic myeloid leukemia; HDAC: Histone deacetylase; ITD: Internal tandem duplication; LL: Lymphoblastic leukemia; MDS: Myelodisplastic syndrome; JMML: Juvenile myelomonocytic leukemia.

* Registration for pediatric indication

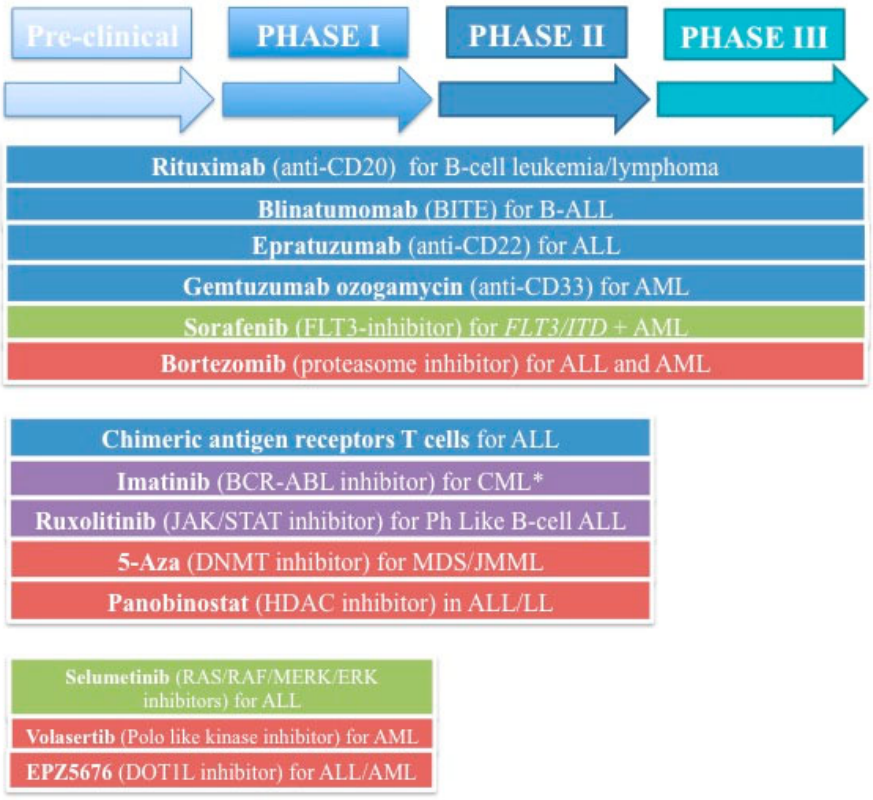

Table 1. Active and near future clinical trials with novel agents in pediatric leukemia

Compound	Target	Indication	Intervention	Population	Phase	Reference
Chimeric antigen receptors directed therapy (CARs)						
CAR-T cells	CD19	Relapsed B-Cell ALL	Single agent	Up to 17 years	II	EudraCT: 2013-003205-25
CAR-T cells	CD19	Relapsed B-Cell malignancy	Single agent	1-30 years	I/II	NCT02772198
CAR-T cells	CD22	Relapsed B-ALL/Lymphoma	Single agent	1-30 years	I	NCT02315612
Cell surface antigens: Immune strategies						
Ofatumumab	CD20	First or second line B-ALL/LL	Obinotuzumab + BFM augmented therapy	12-30 years	II	NCT02419469
Blinatumumab	CD19	HR First Relapse B-ALL	Randomization between blinatumumab or consolidation CT	Up to 17 years	III	NCT02393859
Blinatumumab	CD19	LR, IR, HR Relapse B-ALL	IR and HR: Randomization between blinatumumab or consolidation CT LR: Randomization between backbone CT ± blinatumumab	1-30 years	III	NCT02101853
Denintuzumab	CD19	Relapsed B-ALL or BLe/BLy	Single agent: Weekly or three weekly dosing	From 1 year	I	NCT01786096
Epratuzumab	CD22	SR First Relapse B/T-ALL	Randomization between backbone BFM or UK-ALL R3 CT ± epratuzumab	Up to 17 years	III	NCT01802814
Inotuzumab	CD22	Relapsed B-ALL	Single agent	Up to 17 years	I/II	Launching 2016

Gemtuzumab	CD33	Newly diagnosed AML	Randomization between backbone AML CT ± gemtuzumab	Up to 17 years	III	NCT02724163
Gemtuzumab	CD33	AML/MDS	Post-transplant consolidation GO single agent between day 60 and 180	Up to 30 years	II	NCT02117297
Gemtuzumab	CD33	Newly diagnosed APL	Single arm: GO + ATRA + ATO	From 10 years	II	NCT01409161
BCR-ABL inhibition						
Bosutinib	-	Relapsed/Refractory CML or intolerant to TKIs	Single agent bosutinib	Up to 17 years	I	NTR5501
Small molecules inhibitors of intracellular kinases						
Ruxolitinib	Jak/Stat	Newly diagnosed HR Ph-like B-ALL	Single arm: Backbone induction CT + ruxolitinib	1-21 years	II	NCT02723994
Ruxolitinib / Dasatinib	Jak/Stat	Relapsed Ph-Like B-ALL	3-week window single agent followed by Hyper-CVAD + IP	From 10 years	II	NCT02420717
Selumetinib	Ras/Raf/Mek/Erk	Relapsed ALL	Single arm: Selumetinib and dexamethasone	To be confirmed	I	ECMC
Sorafenib	FLT3	Newly diagnosed HR *FLT3/ITD* + AML	Single arm: Backbone AML CT + sorafenib	Up to 29 years	III	NCT01371981*
Crenolanib	FLT3	Relapsed hematological malignancies FLT3-ITD, TKD mutated	Single arm: Crenolanib and sorafenib	1-39 years	I	NCT02270788
Volasertib	Polo-Like	Relapsed AML	Single arm: Backbone CT + Volasertib	3 months-17 y	I	NCT02722135

Proteasome inhibitors						
Bortezomib	-	Newly diagnosed T-ALL/LL	Randomization between backbone ALL CT ± bortezomib during induction	2-30 years	III	NCT02112916
Bortezomib	-	HR First Relapse B-ALL	Randomization between backbone BFM CT ± bortezomib during induction	Up to 18 years	III	EudraCT: 2012-000810-12
Bortezomib	-	Newly diagnosed AML	Randomization between backbone AML CT ± bortezomib during induction	Up to 29 years	III	NCT01371981*
Carfilzomib	-	Relapsed B-ALL	Single arm: UK R3 Induction backbone CT + carfilzomib	Up to 18 years	I/II	NCT02303821
Epigenetic targeting						
EPZ5676	DOT1L	Relapsed AML/ALL	Single agent EPZ5676	3 months-18 y	I	NCT02141828
Azacitidine	DNMT	Newly diagnosed MDS/JMML	Single agent azacitidine	1 month-18 y	II	NCT02447666
Decitabine	DNMT	Relapsed AML	Single arm: Cytarabine + decitabine	1 month-18 y	I/II	NCT01853228
Vorinostat	HDAC	Relapsed MLLr leukemia	Single arm: Backbone CT + vorinostat + bortezomib	Up to 21 years	II	NCT02419755
Panobinostat	HDAC	Relapsed AML/MDS	Single arm: Panobinostat + fludarabine + cytarabine	Up to 24 years	I	NCT02676323
Panobinostat	HDAC	Relapsed/Refractory ALL/LL	Single arm: Backbone CT + Panobinostat + bortezomib + VSLI	Up to 21 years	II	NCT02518750

ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; APL: Acute promyelocytic leukemia; ATO: Arsenic trioxide; ATRA: All-trans retinoic acid; BFM: Berlin-Frankfurt-Munich BLe: Burkitt leukemia; BLy: Burkitt lymphoma; CARs: Chimeric antigen receptors; CML:

Chronic myeloid leukemia; CT: Chemotherapy; GO: Gemtuzumab-ozogamicin; DNMT: DNA methyltransferase; ECMC: Experimental Cancer Medicine Centre; HDAC: Histone deacetylase; HR: High-risk; IP: Investigational product; IR: Intermediate-risk; ITD: Internal tandem duplication; JMML: Juvenile myelomonocytic leukemia; LL: Lymphoblastic lymphoma; LR: Low-risk; MDS: Myelodysplastic syndrome; MLLr: Mixed lineage leukemia rearranged; SR: Standard-risk; TKD: Tyrosine-kinase domain; TKIs: Tyrosine-kinase inhibitors; UK: United Kingdom; VSLI: vincristine sulfate liposome injection.

* These two studies are part of the same trial (NCT01371981)